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(54) Array of single-stranded DNA immobilised on a solid support

(57) An array of single-stranded DNA probes, each of which comprises at least 75 nucleic acid units, is chemically immobilised on a solid support. The probes may be used to provided a quantitative estimate of the abundance of individual mRNA (or the first strand cDNA corresponding thereto) within a complex mixture thereof, obtained from a biological sample comprising a single cell type or a mixed population of cell types. The probe DNA may be of unknown sequence and may comprise antisense, or sense, strand DNA.

An array of single-stranded DNA, immobilised on a solid support, is prepared by:

(i) provision of samples of double-stranded DNA chemically modified on the sense, or antisense, strand for attachment to the solid support;

(ii) linking the DNA to said support:

wherein, prior to, or after, (ii), the non-modified strand is removed.

The strand, not bound to the support, may be chemically modified, either to assist strand separation or the selective degradation thereof. The double-stranded DNA may be the amplification products of chemically modified primers obtained by the polymerase chain reaction.

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METHODS

This invention relates to methods for preparing arrays of nucleic acids for use in biological screening procedures such as hybridisation assays, with applications in genetic research and diagnostic applications.

Increasing use is being made of arrays of immobilised nucleic acids, particularly arrays of DNA, for genetic research and diagnostic purposes. These arrays consist of a plurality of DNAs organised as a two-dimensional matrix immobilised on an appropriate solid support. Each point in the matrix comprises a DNA element. Each of the DNA elements can be used as a probe to detect complementary sequences in complex mixtures of nucleic acid. This allows parallel determination of the identity and abundance of many DNA species in a single experiment.

Such arrays can be formed on porous membranes such as nitrocellulose using a variety of methods. In the dot-blot or slot-blot technique, a plurality of DNA samples is transferred to membranes by placing the samples into a manifold consisting of an array of pre-formed wells applied to the top of the membrane, and drawing the DNA through the membrane using a vacuum. In another variant of this method, DNA is applied directly to the membrane using an array of pins to transfer DNA onto the membrane surface from DNA samples contained, for example, in the wells of a microtitre plate [Lehrach, H. et al., "Hybridisation Fingerprinting in Genome Mapping and Sequencing" in "Genome Analysis", Vol. 1, Davies, K. E. and Tilghman S. M. (Eds.), Cold Spring Harbor Laboratory Press, New York, 1990, pp38-82; Nizetic, D. et al., Proceedings of the National Academy of Sciences (USA), 1991, 88, 3233-3237].

Alternatively, DNA arrays can be formed on non-porous surfaces such as glass, by either in situ synthesis or direct application. For example, arrays of oligodeoxynucleotides can be assembled by starting with a chemically sensitised glass surface which is protected by a mask, and reacting selected exposed areas with suitably modified nucleotides. By appropriate choice of masks and nucleotide reagents, arrays of synthetic oligodeoxynucleotides of defined sequence can be elaborated at the glass surface [see e.g., Jacobs, J. W. and Fodor, S. P. A., Trends in Biotechnology, 1994, 12, 19-26; Fodor, S. P. A. et al., International Patent Application No. WO 92/10588, published 25 June 1992; Chee, M.

et al., International Patent Application No. WO 95/11995, published 4 May 1995]. An alternative approach has been described by Southern [Southern, E., European Patent No. 0373203B1, published 31 August 1994]. The maximum length of oligonucleotides assembled in these arrays is restricted by the chemistry employed to assemble the oligonucleotides, which in practice are usually no more than 20-25 nucleotides in length.

In another approach, arrays of longer DNA species may be constructed by using robotic micropipetting devices to transfer small, typically nanolitre or smaller quantities of DNA from containers such as 96-well plates to ordered pre-determined positions on a non-porous surface such as a glass microscope slide Each DNA sample is bound at a known position on the microscope slide to constitute one DNA element of the array. Using such apparatus a large number of replica slides can be constructed supporting arrays of thousands of individual DNA elements [Schena M. et al., Science, 1995, 270, 467-470; Shalon, T. D. and Brown, P. O., International Patent Application No. WO 95/35505, published 28 December 1995].

In this latter approach, the DNA samples being transferred to the solid support are typically double-stranded polynucleotide DNA fragments of length greater than 50bp. These DNAs may be obtained from a number of sources, such as cDNA or genomic DNA libraries, and may be of either known or unknown sequence composition.

DNA may be coupled to the solid support by a number of techniques. For example, the DNA may be bound to glass through non-covalent electrostatic interactions with a coating film of a polycationic polymer such as poly-L-lysine [see e.g. Shalon, T. D. and Brown, P. O., International Patent Application No. WO 95/35505, published 28 December 1995]. Alternatively, DNA can be bound covalently to the solid support. There are a number of methods available for covalent linkage of DNA to solid supports, depending on the nature of the support.

Arrays of polynucleotide DNA probes immobilised on solid supports can be used to study the composition of complex mixtures of DNA using hybridisation techniques. In a typical application, a complex mixture of labelled cDNA is hybridised to the DNA array under conditions of appropriate stringency, and unbound material is washed away. The array is then scanned using a detection method capable of sensing the remaining bound labelled cDNA, such as a scanning fluorescent microscope. The intensity of the detected signal at any

given element in the array is a measure of the concentration of the corresponding complementary cDNA in the original complex mixture [Schena M. et al., Science, 1995, 270, 467-470; Shalon, T. D. and Brown, P. O., International Patent Application No. WO 95/35505, published 28 December 1995; Pinkel, D. et al, International Patent Application No. WO 96/17958, published 13th June 1996].

Arrays of immobilised oligonucleotides have been described which use elements containing selected sense, antisense, missense or nonsense sequences at different positions in the array. Such arrays have been used for a number of applications; for example to determine the sequence of DNA [see e.g., Mirzabekov, A. D., Trends in Biotechnology, 1994, 12, 27-32 and references therein; Fodor, S. P. A. et al., International Patent Application No. WO 92/10588, published 25 June 1992; Chee, M. et al., International Patent Application No. WO 95/11995, published 4 May 1995]. In another application, arrays of allele-specific oligonucleotides have been used to detect genetic polymorphisms and determine genotypes [see e.g., Southern, E., European Patent No. 0373203B1, published 31 August 1994; Guo, Z. et al., Nucleic Acids Research, 1994, 22, 5456-546]. However, such arrays have limitations when used to probe complex mixtures of labelled polynucleotide DNA targets such as cDNAs, since any given oligonucleotide may hybridise at a particular stringency to sequences in more than one target DNA. In the case where the sequence of the target DNAs are known, it is possible to design sets of oligonucleotides which will provide a unique hybridisation signal for each gene. Such sets can be combined into one or more elements of an array to provide a hybridisation signal characteristic for any known target [Fodor, S. P. A. et al., International Patent Application No. WO 92/10588, published 25 June 1992; Chee, M. et al., International Patent Application No. WO 95/11995, published 4 May 1995]. However, such an approach cannot be accurately applied where the sequence of the target DNAs is unknown or incomplete. Where the oligonucleotide arrays are constructed by in situ synthetic methods, the addition of an additional target gene requires the whole array to be resynthesised, with considerable cost implications.

In comparison, arrays of longer non-oligonucleotide probe DNAs provide a much higher specificity for hybridisation to target DNAs. However, all such arrays to date have incorporated a mixture of both sense and antisense strands of a particular DNA fragment within each DNA element in the array. In cases where it is desired to detect both strands of

the corresponding target DNA hybridising to a given DNA element, this is not a problem. However, there are applications where it would be advantageous to detect only one strand of any given target DNA in a complex mixture of target nucleic acids. For example, direct detection of a labelled mRNA target requires only an antisense strand DNA probe in each element. Alternatively, direct detection of a first-strand labelled cDNA target, synthesised from an mRNA template by reverse transcriptase, requires only a sense strand DNA probe. In such instances, the presence of the unwanted sense or antisense strand of the probe within each DNA element will reduce signal sensitivity by reducing the number of probe sites available for target hybridisation. It may also increase background signals by hybridising to non-specific target DNAs.

We have now devised methods for preparing single-strand arrays containing elements comprising either sense or antisense polynucleotide DNA probes. These are used to increase the sensitivity of the arrays when used as probes in hybridisation assays with either labelled RNA or labelled single-strand cDNA.

In a first aspect of the invention we provide a method for preparing an array of single-stranded DNA immobilised on a solid support, which method comprises (i) providing samples of double-stranded DNA chemically modified on the sense or antisense strand for attachment to the solid support, and (ii) linking the DNAs to the solid support and, before or after step (ii), removing the non-modified strand whereby an array of single-stranded DNA is provided on the solid support.

In a further aspect of the invention, a second chemical modification is provided on the strand that is not to be bound to the solid support. The purpose of this second chemical modification is to assist in either the separation of the two strands or the selective degradation of the unwanted strand.

The single-stranded DNA preferably comprises DNA molecules containing more than 75 nucleotides such as more than 100 nucleotides or more than 200 nucleotides. Preferred ranges of nucleotides include 100-10,000; 200-10,000 and 300-10,000.

The samples of double-stranded DNA chemically modified on the sense and/or antisense strand are conveniently provided by extension of chemically modified primer(s). Such primer(s) are preferably used as polymerase chain reaction (PCR) primers, whereby the

desired chemical modification(s) are selectively incorporated into the sense and/or antisense strands of the double-stranded DNA.

The primer(s) may be modified at any convenient position(s). Modification(s) are preferably made to the 5' nucleotide of the primer at either the 5' phosphate, the 5' deoxyribose group or the 5' base (adenine, guanine, thymidine or cytosine). In general, the modification involves the addition of a chemical functionality for binding to the solid surface, together with an optional spacer group of appropriate length to improve the accessibility of the probe DNA to the target nucleic acid. Both covalent and non-covalent binding may be used. In one embodiment, the chemical functionality may direct non-covalent binding to the solid surface, for example a biotin moiety which will interact with a streptavidin coating on the solid surface. In an alternative embodiment, the chemical functionality may covalently link the selected DNA strand to the solid surface.

Chemical modification of the DNA may be performed in one or more steps.

It will be appreciated that the sense and antisense strand of each DNA probe may be separated either prior to or after arraying onto the solid support. The separation may involve physical denaturation of the probes using for example heat or alkali, or the enzymic degradation of the unwanted strand for example using an appropriate exonuclease, or a combination of both methods.

In a further aspect of the invention we provide an array of single-stranded probe DNAs, each probe comprising at least 75 nucleotides and immobilised on a solid support. More conveniently each probe comprises at least 100 or 200 nucleotides, such as at least 500 or 1,000 nucleotides. A particular range is 300-10,000 nucleotides. A particular advantage of such an array is that the sequence of the probe DNAs may be unknown. Each probe DNA may be the sense or antisense strand for a given gene sequence. In further particular aspects of the invention every probe DNA in the array is antisense strand DNA or every probe DNA in the array is sense strand DNA.

The array conveniently comprises at least 10 DNA elements, such as at least 100 elements. Further convenient arrays comprise at least 1,000, 10,000 or 100,000 DNA elements.

The invention also provides a method whereby such selected single-strand arrays can be used to provide a quantitative estimate of the abundance of individual mRNAs or their corresponding first strand cDNAs within a complex mixture of such derived from a biological sample comprising a single cell type or a mixed population of cell types. The abundance is determined by measuring the amount of hybridisation between single-strand probe DNA at each element in the array and its complementary strand within the complex mixture of mRNA or cDNA. To detect such hybridisation, a label is incorporated into the mRNA or cDNA molecules in the complex mixture, for example a fluorescent nucleotide. In practising this aspect of the invention, mRNAs are isolated from cells and either directly labelled in vitro or, in a preferred embodiment, converted into first strand cDNAs, in which case the label is introduced on modified nucleotide which is incorporated into the single-strand cDNAs by reverse transcriptase. In this respect, the abundance of any given cDNA species within the population of single-strand cDNAs generated by reverse transcriptase is taken to represent the abundance of the corresponding mRNA within the biological sample.

The labelled cDNAs are hybridised to selected single-strand arrays which contain pairs of elements in which either the sense or antisense strand of each of the polynucleotide probes is immobilised at each element. The amount of immobilised DNA present at each element in the array is controlled such that it is considerably greater than the amount of the corresponding target mRNA or cDNA within the sample applied to the array. Under such conditions, the amount of labelled target nucleic acid (mRNA or cDNA) that remains bound to each element under the hybridisation conditions employed will represent the concentration of each mRNA or cDNA in the original sample. The bound target nucleic acid can be determined using an appropriate detection system capable of measuring the label carried on the target nucleic acid; e.g., a scanning fluorescence microscope [see e.g., Schena M. et al., Science, 1995, 270, 467-470]. The abundance of a particular cDNA (and hence its parental mRNA) may be quantified by comparing the intensity of the specific hybridisation signal, such as fluorescence intensity, at a given sense element to the non-specific hybridisation determined by the signal obtained at its corresponding antisense element. In this way, a

precise quantitation of the absolute abundance of multiple cDNAs can be obtained within a single experiment.

Such single-strand arrays can be readily used to quantify the abundance of single-strand nucleic acid species such as mRNAs or their corresponding first-strand cDNAs in a variety of cell types or populations. The abundance information thus obtained can be used to draw up a quantitative transcript profile describing the expression of a large number of genes within any given cell type or cell population. This information can be used to determine for example which genes are differentially expressed in diseased versus normal tissue, or treated versus untreated tissue, and hence provide valuable information in diagnosing and monitoring disease processes, and in research to identify new treatments to restore the healthy state.

The invention will now be illustrated but not limited by reference to the following detailed description and Example:

i) Definitions

As used herein, the term "animal" is used in its broadest sense to include all members of the animal kingdom.

As used herein, the term "biological sample" encompasses any cell or tissue in any state from any organism which may be selected to provide a source of target nucleic acids.

As used herein, the terms "disease" or "diseased state" refer to any condition which deviates from the normal or standardised healthy state in an organism of the same species in terms of differential expression of the organism's genes. A disease state can be any illness or disorder of genetic or environmental origin which is characterised or may be described by the expression of genes which are either (i) normally silent in the healthy organism but activated in the diseased state as a cause of or in response to the disease, or (ii) normally expressed within some standard range in the healthy organism but over- or under-expressed in the diseased state as a cause of or in response to the disease.

As used herein, the terms "element" or "DNA element" refer to a number of immobilised DNA molecules, which may be either single-stranded or double-stranded, bound

to a solid support at a specific physical location which defines one point within a 2-dimensional matrix constructed from a plurality of such elements.

As used herein, the term "EST" or Expressed Sequence Tag" refers to a partial DNA or cDNA sequence, typically of between 50 and 500 sequential nucleotides, obtained from a genomic or cDNA library prepared from a selected cell, cell type, tissue or tissue type, organ or organism which longer sequence corresponds to an mRNA of a gene found in that library [cf. Adams, M. D. et al., Science, 1991, 252, 1651-1656 and International Application No. PCT/US92/05222, published 7 January 1993]. An EST is generally DNA.

As used herein, the term "gene" refers to the genomic nucleotide sequence from which a cDNA sequence is derived.

As used herein, the term "immobilised" refers to the attachment of probe DNA to a solid support. The attachment may be of a covalent or non-covalent nature and will depend on the nature of the solid support being used.

As used herein, the term "insert" refers to any DNA sequence incorporated within a vector using methods of molecular biology available to anyone ordinarily skilled in the art.

As used herein, the term "oligonucleotide" refers to a molecule containing up to 50 nucleotides, but more typically 20 nucleotides of either DNA or RNA.

As used herein, the term "organism" includes without limitation, microbes, plants and animals.

As used herein, the term "probe" means a DNA species immobilised to a solid support within a DNA element.

As used herein, the term "solid support" refers to any known substrate which is useful for the immobilisation of probe DNA by any available method to enable detectable hybridisation of the immobilised oligonucleotides or polynucleotide DNA sequences to other polynucleotide sequences in a sample. Such useful solid supports include, but are not limited to, paper, nitrocellulose, myelin, glass, silica, nylon, plastics such as polyethylene, polypropylene or polystyrene, or other solid material. In addition, the term "solid support" can refer to gels constructed from such materials as agarose, polyacrylamide, polysaccharide or proteins, which may themselves be overlaid on a further solid surface such as glass or metal, to provide mechanical strength, electrical conductivity or other desired physical

property. Where the solid support is porous, the term "solid support" refers without distinction to a range of pore sizes, depending upon the nature of the system.

As used herein, the term "surface" means any generally two-dimensional structure on a solid support to which the desired probe DNA is attached or immobilised.

As used herein, the term "target" refers to any complex mixture of nucleic acid or any individual component thereof which can be labelled such as to permit its detection by anyone ordinarily skilled in the art.

As used herein, the term "vector" means a DNA sequence capable of maintenance and replication within a host organism. The term "vector" includes, but is not limited to, plasmids such at pBluescript (Stratagene Inc., La Jolla, CA) or bacteriophages such as Lambda UniZAP (Stratagene).

ii) Probe Preparation

The DNA used to generate probes for subsequent arraying may be obtained from a large number of sources. For example DNA fragments may be obtained from a random selection of clones from a DNA library prepared from the organism of interest. In the case of animals such as man or rodents, these clones would preferably be obtained from one or more cDNA libraries. The fragments may also be selected from collections of clones which have been characterised to some extent, for example by partial sequence analysis of the insert DNA or by mapping of the insert DNA to particular chromosomal loci. Such clones may include, but are not limited to the I.M.A.G.E Consortium collection of clones isolated from human or rodent cDNA libraries and characterised by the generation of one or more ESTs for each clone [Lennon, G. et al., Genomics, 1996, 33, 151-152]. In the case of probes derived from bacterial genes, genomic DNA libraries may also be used. Each clone consists of a polynucleotide DNA fragment inserted at a known site within a suitable vector. The vector may for example be a plasmid vector such as pBluescript (Stratagene) or a bacteriophage vector such as Lambda UniZAP (Stratagene).

Individual bacterial clones from selected DNA libraries are cultured in the appropriate liquid medium using standard techniques. A small sample of each culture is used as a source of template DNA for subsequent amplification by PCR, using oligonucleotide primers

complementary to the vector sequences immediately flanking the insert DNA sequence. In this way it is not necessary to know the sequence of the polynucleotide DNA fragment comprising the insert in order to practise the invention.

To prepare a selected single-strand probe, the primer used to direct DNA-polymerase dependent synthesis of the selected strand contains a first chemical modification which will be used to couple that strand to the solid support. Many methods are known in the art whereby nucleic acids can be immobilised on a variety of solid surfaces. In its preferred embodiment, the chemical modification will be incorporated into the 5' nucleotide of the primer at either the 5' phosphate, the 5' deoxyribose group or the 5' base (adenine, guanine, thymidine or cytosine) during synthesis of the oligonucleotide. However, the modification may also be made at other positions within the 5' primer sequence. The modification comprises a chemical functionality for binding to the solid surface, together with a spacer group of appropriate length to improve the accessibility of the probe to the target nucleic acid [see e.g., Maskos, U. and Southern, E. M., Nucleic Acids Research, 1992, 20, 1679-1684 for a discussion of factors influencing linker design]. In one embodiment, the chemical functionality may direct non-covalent binding to the solid surface, for example a biotin moiety which will interact with a streptavidin coating on the solid surface. In an alternative embodiment, the chemical functionality may covalently couple the selected DNA strand to the solid surface. There are a number methods for covalently attaching DNA to solid surfaces through the introduction of various chemical functional groups [see e.g. Ghosh, S. S. and Musso, G. F., Nucleic Acids Research, 1987, 15, 5353-5372; Bischoff, R. et al., Analytical Biochemistry, 1987, 164, 336-344; Guo, Z. et al., Nucleic Acids Research, 1994, 22, 5456-5465]. The precise choice of chemical functionality to be employed will depend on the nature of the solid surface onto which the DNA is to be immobilised. The spacer group may be, for example, a long-chain hydrocarbon of general formula $-(CX_2)n-$ where X may be H or F and n is generally 6-20.

In the case of a sense strand probe the primer used to direct the DNA-polymerase dependent synthesis of the sense strand contains a first chemical modification which will be used to couple that strand to the solid support. In this instance, the corresponding antisense strand primer is either unmodified, or contains a second chemical modification, different from that used in the sense primer. In this embodiment, the modification carried on the antisense

strand is designed to facilitate subsequent strand separation, as described below. For example, where the sense strand primer contains a 5' 6-aminohexyl-phosphodiester group, the antisense strand primer could contain either a 5' phosphate or a 5' biotinylated nucleotide derivative.

In the case of an antisense strand probe the primer used to direct the DNA-polymerase dependent synthesis of the antisense strand contains a first chemical modification which will be used to couple that strand to the solid support. In this instance, the corresponding sense strand primer is either unmodified, or contains a second chemical modification different from that used in the antisense primer. In this embodiment, the modification carried on the sense strand is designed to facilitate subsequent strand separation, as described below. For example, where the antisense strand primer contains a 5' 6-aminohexyl-phosphodiester group, the sense strand primer could contain either a 5' phosphate or a 5' biotinylated nucleotide derivative.

PCR reactions are carried out on DNA obtained from individual clones to obtain the desired number of 5' modified polynucleotide DNA fragments that are to be used as probes in the selected strand arrays. These reactions may be efficiently carried out in high numbers using samples in 96- or 384-well plates and thermocyclers specifically designed to handle such plates. The PCR conditions required for each template will depend upon the precise application and can be readily optimised by anyone ordinarily skilled in the art. The products may be partially purified to remove salts, excess primers and excess nucleotides using an appropriate purification medium such as Sephacryl S-200 which will remove low molecular weight components from the PCR mix.

iii) Preparation of Single-Strand Arrays

The PCR products prepared using modified sense or antisense primers may be separated into sense and antisense strands in two ways. In the first embodiment, the two strands are separated prior to arraying onto the solid support. One desirable method to achieve this is to generate PCR products in which the unwanted strand contains one or more biotinylated nucleotides at the 5' end [Guo, Z. et al., Nucleic Acids Research, 1994, 22, 5456-5465]. The PCR product is bound to streptavidin-coated agarose beads which may be washed to remove other reagents such as salts, primers and free nucleotides, and the two strands then

separated by treatment with 0.1N NaOH for 10 minutes. The beads containing the bound unwanted strand are removed by centrifugation and the supernatant containing the desired non-biotinylated strand is decanted and neutralised to pH7.0 with HCl. This strand is then arrayed and bound to the solid support through the functionality it carries at its 5' end; for example a 5' aminohexyl-phosphodiester group which will couple to glass activated with 1,4-phenylene di-isothiocyanate (DITC).

In a second embodiment, the double-stranded PCR product is arrayed first and the chosen strand coupled to the solid support using the desired chemistry incorporated into the appropriate PCR primer. For example, a strand containing a 5' aminohexyl-phosphodiester group can be coupled to DITC-activated glass. In this embodiment, the unwanted strand will be unable to couple to the solid support because it has been generated using a PCR primer which lacks a 5' amino group. The arrayed double-stranded probe is then denatured, for example using a bath containing 0.1N NaOH, and the unwanted strand washed off. The solid support is then placed in a neutralising bath at pH7.0 to generate the selected strand array.

In a further embodiment, the double-stranded PCR product is arrayed first and the chosen strand linked to the solid support using the desired chemistry incorporated into the PCR primer for that strand. In this embodiment, the unwanted strand is synthesised using a PCR primer which carries an unmodified 5' phosphate group. This strand is then enzymically degraded using a 5' -3' exonuclease, for example lambda exonuclease which cannot attack 5' ends unless they carry a 5' terminal phosphate group ["Current Protocols in Molecular Biology", Ausubel, F. M. et al. (Eds.), Green/Wiley, New York, 1995, pp15.2.5].

In a further embodiment of this invention, the unwanted strand may be removed by a combination of enzymic degradation followed by alkaline denaturation, washing and neutralisation. This combination is particularly effective for probes derived from polynucleotide DNA fragments of lengths approaching 10kb.

Covalently coupling the probe DNA to the solid support at each elemental position through a 5' chemical linker has several advantages. It ensures a robust linkage of DNA to the solid surface which will be resistant to chemical degradation during storage and subsequent procedures, with consequent loss of signal. Importantly, it also provides the maximal amount of single-strand probe DNA which is free to hybridise to target DNA sequences. This is an important advantage over methods that rely on non-specific

electrostatic interactions, such as the binding of probe DNA to poly-L-lysine coated slides, where portions of the DNA probe are complexed with the poly-L-lysine and therefore not available for hybridisation to target DNA. The quantity of single-strand DNA that is arrayed at each element in the array and is free to hybridise to target nucleic acid will vary according to the nature of the solid surface and the chemistry used to link the probes to the solid surface. However it will be present in sufficient quantity to ensure that it is always in excess relative to the concentration of its corresponding labelled target nucleic acid in the sample to be analysed. In this way, the intensity of the resultant hybridisation signal will be proportional to the amount of target nucleic acid present in the biological sample.

In a further aspect of this invention we provide sense strand arrays which comprise a plurality of DNA elements comprising sense strands immobilised on a single solid surface, where the strands in each element are derived from a different polynucleotide DNA fragments and are prepared according to the method described above. In the same way, we provide antisense strand arrays which comprise a plurality of DNA elements comprising antisense strands immobilised on a single solid surface, where the strands in each element are derived from a different polynucleotide DNA fragment and are prepared according to the method described above

In a preferred embodiment of this invention, mixed arrays can be constructed containing pairs of elements comprising either the sense or antisense strand of a given DNA. The pairs of elements do not necessarily have to be arrayed side-by-side within the array. The precise disposition of the two types of element, either within the same array or on different arrays will depend on the precise application for which they are intended.

iv) Hybridisation

Selected single-strand arrays generated as described above may be hybridised to a sample containing a plurality of single-strand target nucleic acids, either mRNAs or preferably, first strand cDNAs that have been isolated from a chosen biological sample and labelled by any of the techniques known to one ordinarily skilled in the art, such as radiolabelling, fluorescent labelling or chemiluminescent labelling [see e.g., Schena M. et al., Science, 1995, 270, 467-470]. In the case of labelled mRNAs, specific hybridisation is

measured using an array of elements comprising antisense single-strand probes, and non-specific hybridisation is measured using an array of the corresponding sense single-strand probes. These two sets of probes may be immobilised on the same or separate solid surfaces as described above. In the case of labelled cDNAs, specific hybridisation is measured using an array of elements comprising sense single-strand probes, and non-specific hybridisation is measured using an array of the corresponding antisense single-strand probes. These two sets of probes may be immobilised on the same or separate solid surfaces as described above.

Hybridisation conditions at the solid support will depend on the nature of the support and the arrayed DNA, but may be defined and optimised using a number of methodologies available to one ordinarily skilled in the art [see e.g., Sambrook, J. et al., "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989]. Preferably, hybridisation takes place under stringent conditions, i.e. those which reveal nucleic acid identities of greater than 95%. However, if desired, other less stringent hybridisation conditions may be selected. Hybridisation of a particular nucleic acid species is detected by measuring the strength of the signal from the labelled target nucleic acid that remains bound its cognate element in the array after washing the array at the particular stringency chosen for the application.

The absolute abundance of a particular single-strand nucleic acid species (be it mRNA or first strand cDNA) in a plurality of nucleic acids may be determined by subtracting the signal at the element in the array corresponding to the non-specific hybridisation from the signal at the element in the array affording the specific hybridisation signal for that particular nucleic acid species. To determine whether the expression of a particular mRNA is altered in some condition, for example a diseased state compared to the normal state, identical arrays are hybridised to labelled samples of target nucleic acids isolated from the diseased and normal biological samples. Differences in the measured abundance can be used to indicate which genes may be involved in the cause, maintenance or progression of the chosen diseased state. The same approach can be used to follow the effects of drug treatment or other investigation of or manipulation of a set of cells or an organism on the expression levels of the genes within the biological sample.

Example 1

Array elements were selected from a set of clones isolated from a human liver cDNA library containing cDNA inserts cloned unidirectionally into a pBluescript vector (Stratagene) between the EcoRI and XhoI sites, such that the 3' end of the insert DNA, including the polyA tail, is located immediately adjacent to the XhoI site. The library was maintained in E. coli strain SOLR™ (Stratagene). The average insert size was 1.5kb. Randomly selected clones were transferred to a 96-deep well microtitre plate and grown in L-broth supplemented with 100 micrograms/ml ampicillin.

To generate sense probes the DNA inserts were amplified using a pair of 24nt primers corresponding to the vector sequences immediately flanking the two restriction sites. The sense primer complementary to pBluescript sequences 5' to the EcoRI site was synthesised with a 6-aminohexyl-phosphodiester at its 5' end. The antisense primer complementary to pBluescript sequences 3' to the XhoI site was biotinylated at its 5' end according to standard procedures [Agrawal, S. et al., Nucleic Acids Research, 1986, 14, 6227-6245]. The PCR reactions with the modified primers were performed directly on small volumes (typically <1 microlitre of overnight culture) of the bacterial cultures in a 96-well thermocycler in final reaction volume of 70 microlitres. Each of the PCR products was purified using a QIAquick™ PCR purification kit (Qiagen Inc., Chatsworth, CA).

Strand separation and removal of the antisense strands was carried out as previously described [Guo, Z. et al., Nucleic Acids Research, 1994, 22, 5456-5465]. The remaining sense strand 5'amino-modified probes were dried down in vacuo and redissolved in 20 microlitres of 100mM sodium carbonate/bicarbonate buffer (pH9.0)

To generate antisense strand probes, the PCR reactions were carried out as described above using an antisense primer with a 5' 6-aminohexyl-phosphodiester group and a biotinylated sense primer. The resulting products were purified and strand-separated as described above.

Pre-cleaned glass microscope slides are treated with 1% 3-aminotrimethoxysilane solution (Aldrich Chemical, Milwaukee, WI), washed, dried and activated with 1,4-phenylene di-isothiocyanate as described by Guo et al. [Guo, Z. et al., Nucleic Acids Research, 1994, 22, 5456-5465]. 2 microlitre samples of either the sense strand or antisense strand DNAs (typically 0.25 - 0.5 micrograms of DNA) are spotted manually onto the microscope slide.

The slide is incubated in a humdified atmosphere at 37_C for 2h, washed with 1% NH_4OH and water and air dried at room temperature.

Glass slides containing arrays of paired elements comprising sense and antisense probes are hybridised with first-strand cDNA prepared by reverse transcription of polyA mRNA isolated from HepG2 cells and labelled with the fluorescent nucleotide analogue dCTP-Cy5 (Amersham International, Chalfont, UK) essentially as described by Schena et al. [Science, 1995, 270, 467-470]. The labelled cDNA (5 micrograms in 7.5 microlitres) is denatured at 95°C. 2.5 microlitres of concentrated hybridisation solution (5 x SSC, 0.1% SDS) is added and the mixture transferred to the glass microscope slide over the array under a cover slip. Hybridisations are carried out in a humidified atmosphere for 12h at 65°C, and the slides washed twice in 0.1 x SSC at 60°C. Fluoresence detection and image reconstruction is carried out as described by Guo et al. [Guo, Z. et al., Nucleic Acids Research, 1994, 22, 5456-5465].

CLAIMS:

1. A method for preparing an array of single-stranded DNA immobilised on a solid support, which method comprises (i) providing samples of double-stranded DNA chemically modified on the sense or antisense strand for attachment to the solid support, and (ii) linking the DNAs to the solid support and, before or after step (ii), removing the non-modified strand whereby an array of single-stranded DNA is provided on the solid support.

2. A method as claimed in claim 1 wherein the single-stranded DNA comprises DNA molecules containing more than 75 nucleotides.

3. A method as claimed in claim 1 or claim 2 wherein the strand that is not to be bound to the solid support is chemically modified to assist strand separation or its selective degradation.

4. A method as claimed in any one of the previous claims wherein the double-stranded DNAs are the amplification products of chemically modified polymerase chain reaction (PCR) primers.

5. An array of single stranded probe DNAs, each probe comprising at least 75 nucleotides and being chemically immobilised on a solid support.

6. An array as claimed in claim 5 wherein each probe comprises at least 200 nucleotides.

7. An array as claimed in claim 5 or claim 6 wherein the probe DNAs are of unknown sequence.

8. An array as claimed in any one of claims 5-7 wherein the probe DNAs are antisense strand DNA.

9. An array as claimed in any one of claims 5-7 wherein the probe DNAs are sense strand DNA.

10. The use of an array as claimed in any one of claims 5-9 to provide a quantitative estimate of the abundance of individual mRNAs or their corresponding first strand cDNAs within a complex mixture of such derived from a biological sample comprising a single cell type or a mixed population of cell types.

The Patent Office

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Application No: GB 9722594.0
Claims searched: 1 to 10

Examiner: Colin Sherrington
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Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.O): C3H(HB4A,HB4B,HB5); G1B(BAG,BAH)

Int Cl (Ed.6): C12Q 1/68

Other: ONLINE: WPI,CLAIMS,DIALOG/BIOTECH

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
A	WO 95/35505 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) -whole document	1, 5
A	Science 1995,270,467-470 -Mark Schena *et al.* "Quantitative Monitoring of Gene Expression with a Complementary DNA Microarray"	1, 5
A	Nucleic Acids Research 1994,22(24),5456-5465 -Zhen Guo *et al.* "Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports"	1, 5
A/X	Analytical Biochemistry 1993,209,63-69 -Shintaro Kawai *et al.* "A Simple Method of Detecting Amplified DNA with Immobilized Probes on Microtiter Wells"	1 / 5 to 10

X Document indicating lack of novelty or inventive step
Y Document indicating lack of inventive step if combined with one or more other documents of same category.
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P Document published on or after the declared priority date but before the filing date of this invention.
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